Correspondence

In situ analysis of embryos with receptor or ligand fusion protein probes

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In situ staining of embryos with receptor or ligand fusion protein probes, initially described in 1994 [1,2], is an increasingly widely used technique to study the ephrins and other molecular families [3]. In a recent letter to Current Biology, Sobieszczuk and Wilkinson [4] describe interesting results showing that overexpression of a ligand can result in masking of its receptor, as assessed with fusion protein probes, emphasizing that the results obtained with such probes always have to be interpreted carefully. As this *in situ* technique is relatively

limitations in comparison with other available methods.

Three other techniques used to study molecular distributions in vertebrate embryos are RNA *in situ* hybridization [5], immunolocalization [6], and reporter gene insertion [7]. It is always important to bear in mind that, by their nature, none of the available techniques can necessarily be assumed to give the biologically 'accurate' expression pattern. Each of them gives a different type of information, and each has unique advantages, as well as limitations.

Briefly, RNA *in situ* hybridization is relatively quick and reliable, and the probes and conditions can be carefully adjusted for a low risk of cross-reactivity. However, it provides no direct information on the pattern or subcellular distribution of proteins, the functional species one is usually interested in. Reporter gene insertion by homologous knock-in or gene trapping can show the RNA expression pattern of the targeted gene and can be highly sensitive and specific. It may not necessarily reflect the natural spatial and dynamic aspects of RNA or protein expression, however. Immunolocalization can show detailed protein distributions and can be highly specific. On the other hand, here too, there are well known limitations. Raising antibodies is time-consuming, expensive, and sometimes unsuccessful. Results must be interpreted with caution, as the antibody may cross-react with multiple proteins. Furthermore, the antibody may bind to, or be affected by, post-translational modifications such as carbohydrate epitopes, and may detect degradation products and other non-functional species.

Ligand or receptor probes are generally made as a fusion protein with an alkaline phosphatase tag [1,2,8] or an immunoglobulin Fc tag [9,10]. They can be produced far more quickly than antibodies. In our experience, production of fusion proteins that retain binding activity

individual receptor or ligand. *In situ* detection procedures are quick and simple, usually taking only a few hours. The fusion probes can be used in the same types of procedure as antibodies, but differ in that they can detect natural ligand–receptor interactions. It is therefore important to recognize that the information gained is qualitatively different. One obvious advantage is that one can find novel binding partners, a feature that has been used to identify a large number of new receptors and ligands. Another unique advantage is that, in the case of receptors that bind to multiple ligands (or vice versa), one can obtain global evidence on the overall distribution of ligands for that receptor. To look at this another way, one can ask a question about the probe — what distribution of ligands can this receptor detect in the embryo? In addition, the technique is presumably selective for functional, available forms of molecules that are capable of ligand–receptor binding.

As with the other techniques, though, one must be cautious in interpreting the results. It may be hard to draw conclusions about any single molecule, since the technique is liable to simultaneously detect multiple binding partners. In addition, formation of pre-existing endogenous receptor–ligand complexes may mask binding sites for the probe. For both of these reasons, it can be informative to compare the results with another technique, such as RNA hybridization.

Sobieszczuk and Wilkinson [4] show that transgenic overexpression of ephrin-A5 can reduce the binding of an ephrin-A5–Fc probe to embryos, providing direct evidence that masking can indeed occur in an embryo. As they point out, it is reassuring that previous studies of ephrins and Eph receptors have generally shown a reasonably good match of fusion-protein binding, when compared with RNA

masking is a mechanism to consider, and indicating that ephrins and their receptors may be less compartmentalized, and more overlapping, than might be concluded based on binding of receptor and ligand fusion proteins [4].

Does this mean that masking can be dismissed as a misleading artifact of the technique? Not necessarily. To give one example, recent studies of retinotectal mapping, by *in vitro* assays, *in vivo* overexpression [11], and gene knockout analysis (D. Feldheim and J.G.F., unpublished observations) indicate that a reduced availability of Eph receptors due to ligand co-expression may be a genuine regulatory mechanism in normal development. Viewed in this light, if fusion protein probes are selective for unmasked sites, this might actually be seen as a useful aspect of the technique that could help in assessing the distribution of available receptor or ligand.

So, the question remains, which of the available techniques for expression pattern analysis most accurately reflects biological reality? The best answer may be 'all of the above'. No one technique necessarily gives the whole picture. Each can provide qualitatively different types of information, and all can help in the quest to understand biological systems.

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The STAS domain — a link between anion transporters and antisigma-factor antagonists

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Anion transporters of the sulfate transporter family are of major interest, as their malfunction is implicated in three human diseases: diastrophic dysplasia/achondrogenesis type IB (DTD) [1,2], Pendred's syndrome (PDS) [3] and congenital chloride diarrhoea (CLD) [4]. The CLD gene is also downregulated in intestinal adenomas and adenocarcinomas [5]. The products of these genes are distinct but related anion transporters that contain 12 transmembrane helices followed by a cytoplasmic domain at the carboxyl terminus. The DTD gene encodes a sulfate transporter [6], the PDS gene a potential iodide-chloride transporter [7] and the CLD gene a chloride–$NaHCO_3^-$ exchanger [8]. Related transporters with the same domain organization, mainly involved in sulfate transport, are present in other eukaryotes and in many bacteria [9].

We describe here an unexpected, statistically significant similarity between the carboxy-terminal cytoplasmic domains of these transporters and the bacterial antisigma-factor antagonists (ASA) typified by *Bacillus subtilis* SPOIIAA. In a PSI-BLAST search [10,11] seeded with the SPOIIAA sequence from *Bacillus stearothermophilus*, with a profile inclusion cut-off of 0.01, the carboxy-terminal domain of the human disease-associated transporters and their eukaryotic and bacterial homologs were detected with random expectation (*E*) values of 10^{-3}–10^{-4} within five iterations. In reciprocal searches initiated with the CLD transporter carboxy-terminal domain, bacterial ASAs were detected with *E* values of 10^{-4}–10^{-6} in the third iteration.

The nuclear magnetic resonance structure of the ASA SPOIIAA [12] provides the structural framework for the emerging domain superfamily. A multiple alignment of this superfamily was constructed using the CLUSTALW program [13] and adjusted using the PSI-BLAST results (Figure 1). The alignment of the carboxy-terminal domains of anion transporters was used for secondary structure prediction-based threading through the Protein Databank (PDB) database using the PHD-TOPITS program [14]. The best hit was the PDB entry for SPOIIAA (PDB code 1AUZ), with a Z-score of 3.1, which strongly suggests a structure similar to that of SPOIIAA. Thus, ASAs and the cytoplasmic portions of anion transporters define a previously undetected, ancient, conserved domain that we named STAS after sulfate transporters and antisigma-factor antagonists.

By mapping the conserved motifs apparent from the multiple alignment (Figure 1) onto the SPOIIAA structure (Figure 2), the conservation was traced largely to the four strands that form the scaffold of the STAS domain. In addition, the turn between the two amino-terminal strands and the long loop between strand 3 and helix 2 are strongly conserved and inserts appear not to be tolerated in these elements (Figures 1,2). Most of the variability is in the loop between helix 1 and strand 3, with α-helical inserts of considerable size seen in some of the anion transporters (Figure 1). A comparison of the alignment with the tertiary structure shows that the carboxy-terminal region of the STAS domain forms a characteristic α-helical handle-like structure (Figures 1,2).

The identification of the STAS domain in the ASAs and the anion transporters provides functional clues for the regulation of anion